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Nucleic Acid Similarities Among *Pseudomonas pseudomallei*, *Pseudomonas multivorans*, and *Actinobacillus mallei*[1]

M. ROGUL, J. J. BRENDLE, D. K. HAAPALA, AND A. D. ALEXANDER

Department of Veterinary Microbiology, Division of Veterinary Medicine, Walter Reed Army Institute of Research Walter Reed Army Medical Center, Washington, D. C. 20012

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Annealing experiments on membrane filters were carried out with deoxyribonucleic acids (DNA) from selected strains of the nomen-species of *Pseudomonas*, *Actinobacillus*, *Chromobacterium*, and *Micrococcus*, with the use of DNA of *Pseudomonas pseudomallei* and *Actinobacillus mallei* as reference materials. Under the usual conditions employed in these experiments, the results were not quantitatively reproducible. Incorporation of dimethylsulfoxide (DMSO) into the incubation medium greatly increased differences in comparative binding. DNA binding in agar matrices was examined in the presence and absence of DMSO at various incubation temperatures. It was found that the greatest specificity, stability, and total binding for DNA containing high amounts of guanine and cytosine occurred in the presence of DMSO. Under the most stringent annealing conditions permitted in agar, DNA species from *P. pseudomallei* and *A. mallei* in the presence of DMSO demonstrated interspecific relative bindings of 76 to 86% when compared to the homologous reactions. The thermal elution midpoints (E_m) of these duplexed interspecific DNA species were quite close to the homologous E_m values. The relative bindings of *P. multivorans* DNA types to either reference DNA ranged between 6 to 27%, and the E_m values were 4 to 7 C less than those for the homologous reactions.

The seventh edition of *Bergey's Manual* assigns the organisms *Pseudomonas pseudomallei* and *Actinobacillus mallei* to the orders *Pseudomonadales* and *Eubacteriales*, respectively. The exclusion of *A. mallei* from the former order seems to have been based primarily on its lack of flagella, sluggish growth on laboratory media, obligate parasitism, and reported serological relationship to *A. lignieresii* and *A. equuli* (18, 36). Wetmore et al. (38) have recently failed to find any significant antigenic relatedness between *A. mallei* and other species of the genus *Actinobacillus*.

In the early part of this century, Whitmore and Krishnaswami (39) and Stanton and Fletcher (34) reported that *P. pseudomallei* and *A. mallei* were similar with respect to pathological and cultural attributes. In more recent studies their conclusions have been substantiated by the demonstration of striking similarities in phage susceptibility and in antigenic, biochemical, and physiological traits (13-15, 25, 28, 32, 37). Most investigators have concluded that both species belong in the family *Pseudomonadaceae*.

As a prelude to projected physiological and genetic experiments, the nucleic acids of these two species were compared. A variety of other bacterial deoxyribonucleic acid (DNA) species were also chosen for comparison with the DNA from *P. pseudomallei* and *A. mallei*. The selection of *Pseudomonas* species was based on recent physiological data (33). The chromobacteria were examined in view of other nucleic acid annealing studies which suggested the possibility that these organisms might be related to some of the pseudomonads (19). Strains of *A. mallei* which were serologically and physiologically similar to *P. pseudomallei* were considered to be logical contenders for interspecies relatedness to the latter species. *A. lignieresii* and *A. equuli* were included because of their purported relatedness to *A. mallei* (*Bergey's Manual*). The DNA species were extracted from these organisms and characterized by composition and homologous and heterologous annealing abilities.

[1] A preliminary report of this study was presented at the 68th Annual Meeting of the American Society for Microbiology, Detroit, Michigan, 5-10 May 1968.

MATERIALS AND METHODS

Organisms and cultural conditions. The strains used and their sources are described in Table 1.

Strains of *P. pseudomallei*, *A. mallei*, *P. multivorans* strains 249 and 382, *P. aeruginosa*, *Chromobacterium janthinum*, *A. lignieresii*, *A. equuli*, and *Micrococcus lysodeikticus* were grown at 37 C. All others were grown at room temperature.

Organisms were grown on either blood-agar or Brain Heart Infusion Agar (Difco) alone or containing 3% glycerol; cells were harvested by scraping them from the surface of the agar.

Nucleic acid extraction. The method of Marmur (26) was followed through the ribonuclease step for the extraction of DNA. After this treatment, sodium lauryl sulfate was added to a final concentration of 1.0%. A 3-mg amount of Pronase (B grade, Calbiochem, Los Angeles, Calif.) was added per ml of the mixture and incubated at 37 C for 4 hr. An equal volume of water-saturated phenol (4 C) was added to the mixture, which was shaken for 0.5 hr in an ice bath. The resulting emulsion was dissociated by centrifugation at 12,000 × *g* at 4 C for 20 min. The DNA in the supernatant fluid was precipitated and extracted by treatment with isopropanol in the presence of acetate as described by Marmur (26). Phenol was removed by washing the DNA successively in ethyl alcohol, acetone, and fresh ether.

TABLE 1. *Organisms used in this study*

Organism	Strain	Per cent GC	Source or reference
Pseudomonas putida B	53	60.7[a]	ATCC 17484 (33)
P. pseudomallei	4845	69.0	ATCC 15682 (37)
P. pseudomallei	1691	69.2	NCTC 1691 (37)
P. pseudomallei	295	69.2	(37)
P. aeruginosa	17	66.9	R. J. Zabransky, Mayo Clinic
P. multivorans	382	67.6[a]	ATCC 17759 (33)
P. multivorans	85	67.6[a]	ATCC 17460 (33)
P. multivorans	249	67.8	ATCC 17616 (33)
P. fluorescens A	197	60.0	(33)
P. fluorescens B	411	61.3[a]	(33)
P. acidovorans	14	66.8[a]	(33)
P. stutzeri	17588	65.0[a]	ATCC 17588 (33)
P. stutzeri	17591	62.1[a]	ATCC 17591 (33)
Chromobacterium janthinum		64.5	WRAIR[b]
C. violaceum		64.8	ATCC 12472
C. amethystinum		62.1	ATCC 6915
C. lividum		61.9	ATCC 12473
Actinobacillus equuli	9347	39.5	(38)
A. lignieresii	207	40.0	(38)
A. lignieresii	33444	34.8	ATCC 10811 (38)
A. lignieresii	1208	38.8	ATCC 15557 (38)
A. mallei	3873	67.2	(37)
A. mallei	4	66.8	(37)
Micrococcus lysodeikticus		71.2	WRAIR

[a] According to M. Mandel (25).
[b] Walter Reed Army Institute of Research.

Radioactive DNA species were prepared from *P. pseudomallei* 4845 and *A. mallei* 3873 grown in 1,200 ml of broth composed of 10.4 g of K_2HPO_4, 4.5 g of KH_2PO_4, 0.1 g of $(NH_4)_2SO_4$, 0.0005 g of $MgSO_4 \cdot 7H_2O$, 2.35 g of Casamino Acids (Difco), and 20 mg of nicotinic acid per liter.

For DNA binding studies on membrane filters (MPF; Millipore Corp., Bedford, Mass.), cytosine-*5-*^3H (6 mc/mmole; Schwarz BioResearch, Inc., Orangeburg, N.Y.) was added to the broth just prior to bacterial inoculation. The *P. pseudomallei* DNA had a specific activity of 12,100 counts per min per μg, the *A. mallei* DNA of 475 counts per min per μg.

For agar-column studies, uracil-*2-*^{14}C (50 mc/mmole; New England Nuclear Corp., Boston, Mass.) was added to the media. The *P. pseudomallei* DNA had a specific activity of 27,300 counts per min per μg, the *A. mallei* DNA of 23,800 counts per min per μg. The cells in radioactive broth were sedimented by centrifugation, and the DNA was extracted as previously described. These DNA species were then sheared and denatured (29). Henceforth, all sheared and denatured radioactive DNA is designated as DNA*.

Determination of DNA base composition. The buoyant densities of nucleic acids were determined in CsCl solutions in a Spinco model E analytical ultracentrifuge. The buoyant densities were converted to per cent nucleotide base composition, expressed as per cent guanine plus cytosine (GC), by the use of the conversion factor of Schildkraut, Marmur, and Doty (30).

Thermal denaturation. Optical density (OD) measurements were obtained in a recording spectrophotometer equipped with thermal spacers. Temperatures were measured with a calibrated thermistor in the optical cell compartment and recorded simultaneously with the OD.

DNA binding on MPF. Annealing tests were performed by the method of Denhardt (10). The nucleic acids were denatured by heating to 100 C in a 1:10 dilution of a standard saline citrate solution (SSC; 0.15 M NaCl plus 0.015 M sodium citrate, *p*H 7.0) for 8 min. Denaturation was determined by sedimenting boundaries at 37,020 rev/min in a Spinco model E analytical ultracentrifuge (9).

Various amounts of denatured DNA were passed through 25-mm, 0.45-μm pore size membrane filters, dried, and stored. DNA retention was determined by three methods: (i) indirectly, by monitoring the effluent of filtered DNA solutions with an ultraviolet (UV) spectrophotometer; (ii) by direct analysis of filters containing radioactive DNA in a liquid scintillation counter, and (iii) by phosphate determinations conducted on these same filters (MPF filters are essentially phosphate free) by the method of Bernhart and Wreath (1). Prior to the phosphate determinations, filters were wet-ashed and heated to dryness in 1 ml of 70% HNO_3 (containing 0.5 mg of $CaCO_3$ per 100 ml) and 0.5 ml of 70% perchloric

acid in tubes cleaned with aqua regia. The assays were usually consistent and comparable.

The Denhardt method was also modified by incorporating 30% (v/v) dimethylsulfoxide (DMSO; Fisher Scientific Co., Silver Spring, Md.) into the incubation media. This seemed to lower the temperature needed for renaturation. In both methods filters loaded with DNA were incubated in PM, the preincubation medium of Denhardt containing 2 × SSC. The filters were removed and placed in specimen vials. One milliliter of PM or 1 ml of PM containing 30% DMSO (PM-DMSO) was added to the vials. The rest of the analysis was carried out by the original method. The amount of radioactivity on the filters was determined in a scintillation counter. The filters were then removed from the scintillation fluid and wet-ashed, and their phosphate content was determined.

DNA binding in agar. Quantitative DNA duplex formation was determined in agar columns. The procedures were similar to those of Bolton and McCarthy (2) and Falkow and Citarella (12). DMSO, at a final concentration of 30%, was used in incubation and elution fluids, because it was found to increase specificity and lower temperatures of denaturation and renaturation. Radioactive DNA was sheared in a Branson sonifier (Heat Systems Co., Millville, N.Y.) to an average molecular weight of 300,000 (29). All DNA species were denatured by heating to 100 C in 0.1 × SSC for 10 min. Unsheared denatured DNA was embedded in Oxoid Ionagar No. 2 (Colab, Chicago Heights, Ill.). The DNA-agar was diced and washed with a 2 × SSC-DMSO solution heated to the temperature used for incubation. To determine whether the DNA had reannealed before it was embedded in agar, 0.3 g of the DNA-agar was incubated with 3 ml of 0.001% acridine orange in McIlvaine's buffer at *p*H 3.8 (5) at 37 C for 30 min and then washed with the buffer until the eluate was colorless. The preparations were observed under a 253-nm UV hand-lamp. Agar containing native DNA fluoresced green, whereas the denatured DNA-agar preparations always showed a bright orange color.

DNA dissolved in 0.1 × SSC was added to agar-trapped DNA in a 1:50 ratio. To maintain consistent solvent conditions, the original agar preparations were considered to be solutions of 2 × SSC containing 30% DMSO (2 × SSC-DMSO). DMSO and SSC of appropriate concentrations were added to the DNA* and DNA-agar mixtures so that the final mixtures were all equivalent to 2 × SSC-DMSO. The volume of fluid added was always numerically equal in milliliters to the weight of the agar in grams.

The resulting slurry was incubated for 16 to 18 hr at 67 C in a silicone-treated 5-ml screw-cap vial. The contents of the vial were transferred to water-jacketed, thermally controlled chromatography columns. The slurry was washed with 200 ml of 2 × SSC-DMSO at the incubation temperature to remove any unbound DNA*. A final wash of 10 ml was collected to monitor leakage. The temperature of the column was then lowered to 44 C, and elution of the specifically annealed DNA* was carried out with 10-ml portions of 0.01 × SSC-DMSO at increments of 2 C up to 74 or 76 C. The eluates were precipitated, filtered, and assayed for radioactivity in a liquid scintillation counter (12).

Control. DNA prepared from *M. lysodeikticus* was used in all annealing experiments to assess the magnitude of nonspecific retention and aggregation ("background noise"). Although this bacterium has DNA of high GC content, similar to that of *P. pseudomallei* and *A. mallei*, it differs so greatly from these organisms in phenotypic respects that little, if any, specific annealing of its DNA could be expected to occur with the DNA of the two latter organisms.

RESULTS

Retention of DNA and duplex formation on MPF. The retention of denatured DNA species on MPF was usually consistent for each DNA preparation and lot of filters. Retention ranged from 16 to 77% of input for the various DNA preparations. Specific binding of *P. pseudomallei* DNA* in 2 × SSC appeared to occur optimally at 72 C in comparative annealing tests with homologous and *M. lysodeikticus* DNA species. However, in additional comparative tests under these conditions (Table 2), "background noise" was found to be high and the amount of DNA* bound to DNA immobilized on filters was not reproducible quantitatively.

Legault-Démare et al. (23) showed that the temperature of renaturation of DNA on membrane filters could be lowered if 30% (v/v) DMSO was incorporated into the incubation solution. For this reason, 30% DMSO was used in the Denhardt method. The most specific binding seemed to occur at 60 C. The results were still not reproducible but "background noise" was considerably reduced (Table 2). Determinations of radioactive DNA and phosphate showed that the DNA species were eluting from the filters in unpredictable amounts during overnight incubation. Nonetheless, the introduction of DMSO into the incubation fluid resulted in outstanding differential binding abilities among the various DNA species. Similar results occurred when *A. mallei* DNA* was used. It was then apparent that the MPF methods could be utilized as screening procedures and that three species, *P. pseudomallei*, *A. mallei*, and *P. multivorans*, have nucleic acids with some base sequence complementarity. To evaluate the similarities of these DNA species, the agar-column technique (12) was used in an attempt to obtain reproducible measurements.

Thermal denaturation studies of native DNA in various solvents. The required experimental conditions for eluting annealed DNA* from DNA in agar was determined by dissolving *P. pseudomallei* 4845 native DNA in solvents of different

TABLE 2. *Quantitative determinations of nucleic acid annealing on membrane filters (Millipore)*[a]

Strain	Relative per cent annealed with *P. pseudomallei* 4845 DNA*[b]			
	Preincubation medium		Preincubation medium—30% DMSO	
	Trial 1	Trial 2	Trial 1	Trial 2
P. pseudomallei 4845	100	100	100	100
P. pseudomallei 1691			122	123
P. pseudomallei 295	79.1	91.9	120	347
A. mallei 3873				132
A. mallei 4			170	
P. aeruginosa	13.9	6.5	0.7	0.6
P. multivorans 382	40.5	51.6	19.7	18.4
P. fluorescens A	5.6	7.1	0.1	0.1
P. fluorescens B			0.4	0.05
P. acidovorans	7.0	6.3	1.2	0.8
P. putida	4.1		0.5	1.0
C. janthinum			1.3	0.2
C. violaceum			2.5	1.7
C. amethystinum			1.8	1.7
C. lividum			2.3	2.8
A. lignieresii 33444	0.8	1.2	0.4	0.3
A. equuli	1.6	1.0		
M. lysodeikticus	9.3	19.9	0.4	1.3

[a] Annealing in preincubation medium (PM) was performed at 72 C; PM-30% DMSO at 60 C overnight. Ratio of DNA* to DNA originally retained on filter is 1:50.

[b] Relative per cent annealed is derived by determining counts per min per μg of DNA retained on filter after incubation and dividing by counts per min per μg on homologous reaction filter and then multiplying by 100.

salt concentrations with and without DMSO. The solutions were heated gradually and changes in optical density were recorded (Table 3). The temperatures of initial and final rise in hyperchromicity were considered to be analogous to the temperatures of initial and complete denaturation of native DNA and comparable to conditions necessary for the elution of annealed DNA* in an agar matrix. Because the agar gel dissolves around 75 C, 0.01 × SSC containing 30% DMSO was chosen as the eluant for the duplexes formed in the agar. The denaturation temperatures in this solvent ranged over 14 C and the approximate midpoint of thermal denaturation (T_m) was 58 C.

Conditions affecting DNA duplex formation in agar. Table 4 and Fig. 1 illustrate some of the characteristics of binding and elution patterns of *P. pseudomallei* 4845 DNA* with *P. pseudomallei* 4845 and *M. lysodeikticus* DNA-agar. The degree of annealing was determined in 2 × SSC as well as in 2 × SSC-DMSO at various incubation temperatures. In both solvents the greatest binding occurred during incubation at 60 C.

In 2 × SSC at 60 C, large amounts of bound DNA* in the homologous (Fig. 1A) and heterologous (Fig. 1B) reactions eluted at very low temperatures. In both reactions, increased incubation temperatures apparently resulted in decreased amounts of DNA* which was bound. The thermal elution midpoint (E_m) values of the homologous reactions increased with increasing incubation temperatures, but did not reach the T_m values of native DNA. At 70 C there was a very noticeable reduction in annealing with *M. lysodeikticus* DNA-agar (1.9%), whereas the homologous binding remained elevated (40.9%).

The incubation mixture of 2 × SSC containing 30% DMSO appeared to be much more satisfactory for obtaining optimal DNA binding. In the homologous reaction, incubation between 60 and 75 C resulted in relatively large amounts of DNA binding and the E_m values approximated the T_m values of native DNA. However, quantitation of binding at 75 C was hindered because the agar melted. Integral plots of per cent DNA* eluted were all sigmoid with narrow transitions (Fig. 1C). This is usually characteristic of tightly duplexed structures. *P. pseudomallei* DNA* did not anneal with *M. lysodeikticus* DNA-agar to any appreciable extent in 2 × SSC-DMSO (Table 4, Fig. 1D).

At 60 C, the most permissive temperature of annealing, only the DNA* bound in the homologous reaction in 2 × SSC-DMSO eluted in a normal distribution around the E_m (histograms of Fig. 1).

The most stringent conditions for duplex formation were sought to minimize adventitious binding and nonspecific aggregation, which might have led to false conclusions of genetic relatedness. In either solvent system, highly specific annealing occurred between 65 and 70 C. Consequently, all further annealing was carried out in 2 × SSC-DMSO at 67 C.

TABLE 3. *Range of Pseudomonas pseudomallei DNA denaturation temperatures as determined by hyperchromicity in various solvents*

Solvent	Temp of	
	Initial hyperchromicity	Final hyperchromicity
	C	C
0.01 × SSC	56	77
0.1 × SSC	72	88
0.01 × SSC-30% DMSO	51	65
0.1 × SSC-30% DMSO	64	76

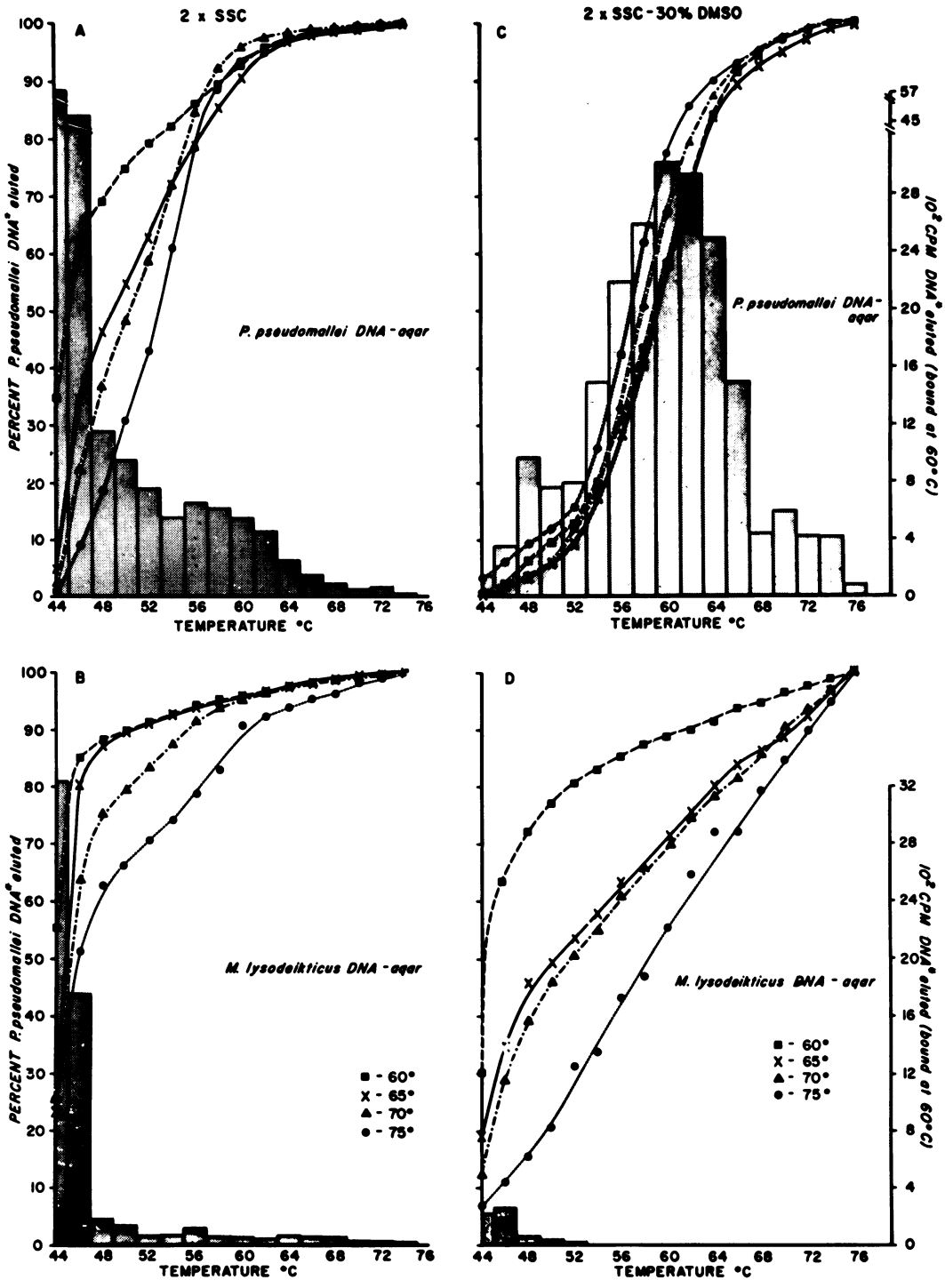

FIG. 1. *Effect of incubation temperatures and solvents on elution of Pseudomonas pseudomallei 4845 DNA* from homologous and heterologous DNA-agar preparations. Cumulative percentages of DNA* eluted are presented for each temperature of incubation. Histograms are only for incubations of 60 C. This was the most permissive temperature for total binding. When compared with Table 4 these histograms indicate the relative trend in which DNA* is bound and eluted.*

Quantitative annealing of selected DNA species. The results obtained with the bacterial strains examined are listed in Table 5. *P. stutzeri* strains were included in these experiments because their colonial appearance is superficially similar to that of *P. pseudomallei*. In general, the specific annealing between DNA types of *P. pseudomallei* and *A. mallei* DNA types was greater than 70% of that obtained in the homologous reactions. The E_m values of these DNA types were very close to those of the homologous reactions.

Strain 295 of *P. pseudomallei* seemed to be somewhat aberrant as shown by the relatively low ability of its DNA to bind to DNA* of the *P. pseudomallei* reference strain. The quantity annealed and the duplex stability were both slightly depressed.

The DNA types of *P. multivorans* strains gave comparative binding values between 6.4 and 17.9% when tested with *P. pseudomallei* 4845 DNA*, and between 9.8 and 27.2% when tested with *A. mallei* 3873 DNA*. The shapes of the elution profile curves of *P. pseudomallei* and *A. mallei* DNA* types eluted from the *P. multivorans* DNA-agar were skewed toward the lower temperatures. Therefore, the curves expressing

TABLE 4. *Per cent bound and elution midpoints of DNA* and DNA-agar preparations in different solvents at various annealing temperatures*

Renaturing temp	Percent bound of *P. pseudomallei* DNA[a]					
	2 × SSC			2 × SSC-30% DMSO		
	P. pseudomallei DNA-agar	E_m[b] temp	*M. lysodeikticus* DNA-agar	*P. pseudomallei* DNA-agar	E_m temp	*M. lysodeikticus* DNA-agar
C	%	C	%	%	C	%
60	55.9	44.5	19.4	70.4	59	2.3
65	49.7	48.8	8.3	54.1	59.2	1.3
70	40.9	50.2	1.9	41.4	58	1.2
75	32.8	52.9	0.4	17.5	56.8	0.6

[a] Ratio of DNA* to DNA in agar is 1:50.

[b] Elution midpoint temperatures are only for *P. pseudomallei* DNA in agar. E_m values were obtained in 0.01 × SSC containing 30% DMSO.

TABLE 5. *Comparative binding abilities of Pseudomonas pseudomallei and Actinobacillus mallei DNA species to homologous and heterologous DNA species in agar matrices*

Source of immobilized DNA	*P. pseudomallei* 4845 DNA* Heterologous binding × 100 / Homologous binding	E_m	*A. mallei* 3873 DNA* Heterologous binding × 100 / Homologous binding	E_m
	%	C	%	C
Pseudomonas pseudomallei 4845	Homologous[a]	57.4 ± 1.6[b]	83.1 ± 1.7	56.8 ± 0.4
P. pseudomallei 295	76.7 ± 0.7[b]	54.8 ± 0.3	85.0 ± 3.0	55.1 ± 0.4
P. pseudomallei 1691	91.8 ± 4.5	57.4 ± 0.3	85.9 ± 0.1	56.2 ± 0.6
Actinobacillus mallei 3873	76.2 ± 7.0	55.9 ± 1.6	Homologous	56.0 ± 1.2
A. mallei 4	76.3 ± 1.9	56.9 ± 1.4	86.7 ± 5.2	56.5 ± 1.0
Pseudomonas multivorans 249	17.9 ± 0.8	52.3 ± 1.0	27.2 ± 2.4	51.7 ± 0.5
P. multivorans 85	10.9 ± 1.4	51.8 ± 1.8	14.9 ± 0.4	50.8 ± 1.0
P. multivorans 382	6.4 ± 1.6	53.8 ± 0.7	9.8 ± 3.6	50.0 ± 1.4
P. stutzeri 17588	1.8[c]			
P. stutzeri 17591	2.2 ± 0.1			
Micrococcus lysodeikticus	0.8 ± 0.7		0.4 ± 0.1	

[a] For *P. pseudomallei*, 35,346 counts per min were obtained for the homologous binding with DNA containing 27,300 counts per min per μg of ^{14}C DNA; for *A. mallei*, 39,639 counts per min with DNA of 23,800 counts per min per μg of ^{14}C DNA. Ratio of DNA* to DNA in agar is 1:50.

[b] Results are expressed as ratio of (heterologous binding to homologous binding) × 100, with each the mean and standard deviation of at least two determinations.

[c] Only one test.

the per cent of DNA eluted as a function of temperature were not typically sigmoidal and show an elevation at lower temperatures.

P. stutzeri DNA types did not demonstrate any significant binding with *P. pseudomallei* DNA*.

DISCUSSION

In this study, attempts to measure DNA annealing on membrane filters did not give satisfactory quantitative results. Other investigators have reported similar inconsistencies (most often observed as a consequence of irregular leaching of the immobilized DNA from the filters). They have attributed their difficulties to variations in the type of filters used (differences in composition), variation in production lots from the same manufacturer, uneven wetting abilities of the filters, and peculiarities of the specific DNA preparation (3, 4, 16, 21, 23, 31, 40). In our studies, it appeared that the leaching of DNA from the filters during incubation was increased by the high temperature employed for specific annealing.

Legault-Démare et al. (23) found that use of 30% DMSO in the incubation fluid lowered the temperature at which optimal renaturation of DNA occurred on membrane filters.

It was hoped that the use of DMSO would not only lower the temperature needed for specific annealing but concurrently lessen the amount of DNA leaching from the filters. The DNA still eluted in unpredictable amounts and did not yield consistent quantitative results. Nonetheless, the use of DMSO greatly sharpened the differences in comparative binding of heterologous DNA species so that the bindings among DNA species of *P. pseudomallei*, *P. multivorans*, and *A. mallei* were highly emphasized. In terms of DNA base composition (Table 1) and of DNA binding (Table 2), the strains of *A. lignieresii* and *A. equuli* examined could not be genetically related to either *A. malei* or *P. pseudomallei*. In the preliminary experiments, the DNA species from the chromobacteria, *P. aeruginosa*, *P. fluorescens*, *P. acidovorans* and *P. putida* did not bind to *P. pseudomallei* or *A. mallei* DNA* types to any greater extent than the control DNA from *M. lysodeikticus*, and were not tested further.

The species *P. pseudomallei*, *A. mallei*, and *P. multivorans*, which did show significant DNA base sequence complementarity in the preliminary experiments, were investigated in more detail by the agar column technique. In the agar column technique, the major criteria for specific binding were (i) that the renatured homologous fragments should approximate the behavior of native DNA under similar circumstances and (ii) that the conditions of annealing and elution should not conflict with the quantitation of binding.

Since DMSO seemed to aid in the reduction of nonspecific binding, the conditions employed by Legault-Démare et al. (23) on membrane filters were transposed and investigated in agar matrices. *P. pseudomallei* DNA* and homologous single-stranded DNA-agar were annealed in 2 × SSC at various temperatures and compared to annealing in 2 × SSC-DMSO. *M. lysodeikticus* DNA was used as the nonrelated control.

Elevation of the renaturation temperature in 2 × SSC media resulted in higher E_m values, which were indicative of more stabilized duplex structures (Table 4, Fig. 1A). Even so, these E_m values were consistently lower than the T_m of native DNA in the same elution solvent. When the percentages of DNA* eluted were plotted at the various incubation temperatures, the resulting curves were more sigmoid in shape as the incubation temperatures were increased.

In comparison, the E_m values obtained in 2 × SSC-DMSO media remained consistently high and approximated the T_m of native DNA. In these cases, curves derived from plots of percent DNA* eluted were sigmoid in shape and formed a sharp transition of elution (Fig. 1C).

The combination of low incubation temperatures and 2 × SSC allowed the apparent annealing of *P. pseudomallei* DNA* to *M. lysodeikticus* DNA-agar. If only the quantities annealed were considered, a false estimation of homology would have been deduced. This was avoided by demonstrating that these complexes were highly unstable. Histograms constructed from DNA* elution values after annealing at 60 C did not form a normal distribution and no E_m values could be determined, since more than 50% of the DNA* was eluted in the first two washes (Fig. 1B). This was decidedly not nucleotide complementarity, which could be correlated with genetic relatedness. In 2 × SSC-DMSO at this same incubation temperature, no significant affinity (amount annealed and stability of duplex) was demonstrable (Fig. 1D). The use of DMSO in the incubation medium resulted in greater affinity in homologous bindings and reduced DNA aggregation and nonspecific binding (Table 4; Fig. 1).

In both solvents, it appeared that the amounts of homologous DNA* which had annealed decreased as the incubation temperature increased. In most bacterial systems this has been characteristic of heterologous rather than homologous DNA bindings (7). This reduction was most probably due to a random but consistent leach-

ing of DNA from the agar matrix (27). It is also possible that adenine- and thymine (AT)-rich fragments were preferentially denatured and eluted, whereas GC-rich areas were annealed (6). If the latter condition had occurred in 2 × SSC-DMSO, we would have expected the elution histograms (not shown) to have different shapes and the E_m values to be appreciably increased with the incubation temperature. This was not the case, and we have excluded the probability of preferential AT melting in this solvent.

In nucleic acid studies, DMSO has been mainly used as a denaturant. The cause of this denaturation has been variously attributed to the ability of DMSO to disrupt H-bonds, increase solubility and stability of single-stranded DNA (20), and increase the London dispersion forces between the DNA bases (17). Similar reagents are thought to decrease ion solvation and increase the hydrophobic character of the solvent (24).

In light of these properties, it is difficult to reconcile the denaturing abilities of DMSO with its facility for renaturation. However, since denaturation of DNA containing large amounts of GC results in a high degree of intrachain base pairing, it may be that DMSO aids in breaking intrastrand bonds as a prerequisite to interstrand H-bonding (11, 35).

Johnson and Ordal (22) and others (7, 8, 27) have recently shown how false assumptions of genetic relatedness can be derived from quantitations of annealed DNA species under nonstringent conditions, especially those DNA species which contain high amounts of GC. For this same reason, and in consideration of projected genetic studies, we chose stringent conditions of annealing which would demonstrate only highly specific regions of base sequence compatibility.

In Table 5, the data are presented to emphasize the relative amounts of DNA bound and duplex stability (E_m), thus directing attention to regions of nonhomology within segments of duplexed strands. The stabilities and relative bindings of DNA species from *A. mallei* and *P. pseudomallei* are in good agreement with the phenotypic and phage typing similarities noted by others (13–15, 25, 28, 32, 37). Based on this evidence, the placing of these organisms in the same taxo- and genospecies is probably justified. The most aberrant of these strains, *P. pseudomallei* 295, is a motile organism whose DNA seemed to have a greater affinity with the DNA of *A. mallei* 3873 than with that of *P. pseudomallei* 4845. It would be interesting to speculate that more testing would elucidate a spectrum of organisms confined within the genetic limits of these two species.

Redfearn et al. (28) have noted some striking physiological similarities between *P. multivorans*, *P. pseudomallei*, and *A. mallei*. The present study serves to substantiate their findings. Although the amounts of *P. multivorans* DNA types annealed with *P. pseudomallei* and *A. mallei* DNA species were relatively small (6 to 27%), the stability of the duplexes ascertained that those segments which did bind had nucleotide sequence complementarity.

On the basis of criteria set forth in *Bergey's Manual* and by Stanier et al. (33), *P. pseudomallei* and *P. multivorans* appear to be correctly placed in the genus *Pseudomonas*. Since *A. mallei* shares phenotypic and genetic similarities with both these organisms, it should be placed within the family *Pseudomonadaceae* and most probably assigned to the genus *Pseudomonas*.

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